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(54) Title: AN IMPROVED METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

(57) Abstract

The present invention addresses the need to improve the yields of viral vectors when grown in cell culture systems. In particular, it has been demonstrated that for adenovirus, the use of low–medium perfusion rates in an attached cell culture system provides for improved yields. In other embodiments, the inventors have shown that there is improved Ad–p53 production with cells grown in serum–free conditions, and in particular in serum–free suspension culture. Also important to the increase of yields is the use of detergent lysis. Combination of these aspects of the invention permits purification of virus by a single chromatography step that results in purified virus of the same quality as preparations from double CsC1 banding using an ultracentrifuge.

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AN IMPROVED METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

1. Field of the Invention

The present invention relates generally to the fields of cell culture and virus production. More particularly, it concerns improved methods for the culturing of mammalian cells, infection of those cells with adenovirus and the production of infectious adenovirus particles therefrom.

2. Description of Related Art

Adenoviral vectors, which carry transgenes that can be transcribed and translated to express therapeutic proteins, are currently being evaluated in the clinic for the treatment of a variety of cancer indications, including lung and head and neck cancers. As the clinical trials progress, the demand for clinical grade adenoviral vectors is increasing dramatically. The projected annual demand for a 300 patient clinical trial could reach approximately 1.08×10^{16} viral particles.

Traditionally, adenoviruses are produced in commercially available tissue culture flasks, "cellfactories," or RB. Virus infected cells are harvested and subjected to multiple freeze-thaws to release the viruses from the cells in the form of crude cell lysate. The produced crude cell lysate (CCL) is then purified by multiple CsCl gradient ultracentrifugation steps. The typically reported virus yield from 100 single tray cellfactories is about 1×10^{14} viral particles. Clearly, it becomes unfeasible to produce the required amount of virus using this traditional process. New scaleable and validatable production and purification processes have to be developed to meet the increasing demand.

The purification throughput of CsCl gradient ultracentrifugation is so limited that it cannot meet the demand for adenoviral vectors for gene therapy applications. Therefore, in order to achieve large scale adenoviral vector production, purification methods other than CsCl gradient ultracentrifugation have to be developed. Reports on the chromatographic purification of viruses are very limited, despite the wide application of chromatography for the purification of recombinant proteins. Size exclusion, ion exchange and affinity chromatography have been evaluated for the purification of retroviruses, tick-borne encephalitis virus, and plant viruses with varying degrees of success (Crooks, *et al.*, 1990; Aboud, *et al.*, 1982; McGrath *et al.*, 1978, Smith and Lee, 1978; O'Neil and Balkovic, 1993). Even less research has been done on the chromatographic purification of adenovirus. This lack of research activity may be partially attributable to the existence of the effective, albeit non-scalable, CsCl gradient ultracentrifugation purification method for adenoviruses.

Huyghe *et al.* (1996) have reported adenoviral vector purification using ion exchange chromatography in conjunction with metal chelate affinity chromatography. Virus purity similar to that from CsCl gradient ultracentrifugation was reported. Unfortunately, only 23% of virus was recovered after the double column purification process. Process factors that contribute to this low virus recovery are the freeze/thaw step utilized by the authors to lyse cells in order to release the virus from the cells and the two column purification procedure.

Clearly, there is a demand for an effective and scaleable method of adenoviral vector production that will result in a high yield of product to meet the ever increasing demand for such products. Recently Blanche *et al* in WO 98/00524 , based on USSN 60/026,667, describe adenoviral production methods that are useful as descriptive art. PCT publication No. WO 98/00524 and USSN 60/026,667 are specifically herein incorporated by reference for their description of techniques for production and purification of recombinant adenovirus.

SUMMARY OF THE INVENTION

The present invention describes a new large scale process for the production and purification of adenovirus. This new production process offers not only scalability and validatability but also virus purity comparable to that achieved using CsCl gradient ultracentrifugation.

The present invention relates to a process for preparing large scale quantities of adenovirus. Indeed, it is believed that very large quantities of adenovirus particles can be produced using the processes of the present invention, quantities of up to about 1×10^{18} particles, and preferably at least about 5×10^{14} particles. This is highly desirable, as there are currently no techniques available to produce the very large, commercial quantities of adenovirus particles required for clinical applications at the high level of purity needed.

In one embodiment, the process generally involves preparing a culture of producer cells by seeding producer cells into a culture medium, infecting cells in the culture after they have reached a mid-log phase growth with a selected adenovirus (*e.g.*, a recombinant adenovirus), and harvesting the adenovirus particles from the cell culture. This is because it has surprisingly been discovered by the inventors that maximal virus production is achieved in the producer cells when they are infected in the later part of log phase growth and prior to stationary growth. Preferably, the adenovirus particles so obtained are then subjected to purification techniques either known in the art or set forth herein.

In certain preferred embodiments of the present invention, therefore, the producer cells are infected with adenovirus at between about mid-log phase and stationary phase of growth. The log phase of the growth curve is where the cells reach their maximum rate of cell division (*i.e.* growth). The term mid-log phase of growth refers to the transition mid-point of a logarithmic growth curve. Stationary phase growth refers to the time on a

growth curve (*i.e.* a plateau) in which cell growth and cell death have come to equilibrium.

In even more preferred embodiments, the producer cells are infected with the adenovirus during or after late-log phase of growth and before stationary phase. Late-log phase is defined as cell growth approaching the end of logarithmic growth, and before reaching the stationary phase of growth. Late-log phase can typically be identified on a growth curve as a secondary or tertiary point of inflection that occurs as the log-growth phase slows, approaching stationary growth.

In a preferred embodiment of the present invention, the producer cells are seeded into the cell culture medium using an essentially homogeneous pool of cells. The inventors have surprisingly discovered that the use of a homogeneous pool of cells for seeding can provide much improved confluency and cell density as well as better maturation of the virus, which in turn provides for larger production quantities and ultimate purity of the virus recovered. Indeed, seeding through the use of separate rather than homogeneous cell populations, for example from individual cell culture devices used in the cell expansion phase, can result in uneven cell density, and therefore uneven confluency levels at the time of infection. It is believed that the use of a homogeneous cell pool for seeding overcomes these problems.

In another preferred embodiment of the present invention, the culture medium is at least partially perfused during a portion of time during cell growth of the producer cells or following infection. Perfusion is used in order to maintain desired levels of certain metabolites and to remove and thereby reduce impurities in the culture medium. Perfusion rates can be measured in various manners, such as in terms of replacement volumes/unit time or in terms of levels of certain metabolites that are desired to be maintained during times of perfusion. Of course, it is typically the case that perfusion is not carried out at all times during culturing, *etc.*, and is generally carried out only from time to time during culturing as desired. For example, perfusion is not typically initiated

until after certain media components such as glucose begin to become exhausted and need to be replaced.

The inventors have discovered that low perfusion rates are particularly preferred, in that low perfusion rates tend to improve one's ability to obtain highly purified virus particles. The inventors prefer to define perfusion rate in terms of the glucose level that is achieved or maintained by means of the perfusion. For example, in the present invention the glucose concentration in the medium is preferably maintained at a concentration of between about 0.5 g/L and about 3.0 g/L. In a more preferred embodiment, the glucose concentration is maintained at between about 0.70 g/L and 2.0 g/L. In a still more preferred embodiment, the glucose concentration is maintained at between about 1.0 g/L and 1.5 g/L.

Also in certain preferred embodiments, the inventors prefer to recirculate the cell culture media while carrying out processes in accordance with the present invention, and even more preferably, the recirculation is carried out continuously. Recirculation is desirable in that it affords a more even distribution of nutrients throughout the cell growth chamber.

In certain other embodiments, the cells are seeded into the culture medium and allowed to attach to a culture surface for between about 3 hours and about 24 hours prior to initiation of medium recirculation. Attachment of cells to a cell surface generally allows for a more consistent and uniform cell growth and higher virus production rate, which in turn allows for the production of higher quality virus. It has been found by the inventors that recirculation can sometimes impede consistent and uniform cell attachment, and that ceasing recirculation during cell attachment phases can provide significant advantages.

With respect to seeding, in a preferred embodiment of the present invention, the cell culture medium is seeded with between about 0.5×10^4 and about 3×10^4 cells/cm^2,

and more preferably with from about 1-2 x 10^4 cells/cm^2. The reason for this is that it has been found that in order to achieve maximal cell expansion and growth, it is most preferable to inoculate the selected growth chamber with a lower number of cells that one might typically use in other cell growth situations. The inventors have found that higher numbers of cells used in the cell inoculation step results in a cell density that is too high and can result in an over-confluence of cells at the time of viral infection, thus lowering yields. It is well within one of skill in the art to determine that in other types of cell culturing systems, similar optimization of the seeding density for a particular system could easily be determined. Nevertheless, in a particularly preferred embodiment, the cell culture medium is seeded with between about 7.5 x 10^3 and about 2.0 x 10^4 cell/cm^2. In an even more preferred embodiment, the cell culture medium is seeded with between about 9 x 10^3 and 1.5 x 10^4 cells/cm^2.

In another preferred embodiment of the present invention, the harvested adenovirus is purified and placed in a pharmaceutically acceptable composition. A pharmaceutically acceptable composition is defined as one that meets the minimal safety required set forth by the FDA or other similar pharmaceutical governing body, and can thus be administered safely to a patient. The present invention provides processes for the purification of the adenovirus. For example, the adenovirus is purified by steps that include chromatographic separation. While more than one chromatography step can be used in accordance with the present invention to purify the adenovirus, this will often result in significant losses in terms of yield. Thus, the inventors have discovered that surprising levels of purity can be achieved where only a single chromatography step is carried out, particularly where that chromatography step is carried out using ion-exchange chromatography. Ion-exchange chromatography is an excellent choice for purification of adenovirus particles due to the presence of a net negative charge on the surface of adenoviruses at physiological pH, permitting high purity isolation of adenovirus particles.

In particular embodiments of the present invention, the recombinant adenovirus is a replication-deficient adenovirus encoding a therapeutic gene operably linked to a

promoter. A replication deficient adenovirus carrying a therapeutic gene linked to a promoter allows the controlled expression of the therapeutic gene by activating the promoter. The precise choice of a promoter further allows tissue specific regulation and expression of the therapeutic gene. In particular embodiments, the promoter is an SV40 IE, RSV LTR, β-actin, CMV-IE, adenovirus major late, polyoma F9-1, or tyrosinase promoter.

In other embodiments the replication deficient adenovirus is lacking at least a portion of the E1 region of the adenoviral genome. Replication deficient adenoviruses lacking a portion of the E1 region are desired to reduce toxicity and immunologic reaction to host cells. In another embodiment of the present invention, the producer cells complement the growth of replication deficient adenoviruses. This is an important feature of producer cells required to maintain high viral particle number of the replication deficient adenovirus. In certain such embodiments, the producer cells are 293, PER.C6, 911 or IT293SF cells. In a preferred embodiment, the producer cells are 293 cells. This allows

In a preferred embodiment of the present invention it is contemplated that the recombinant adenovirus encodes a therapeutic recombinant gene. For example, the therapeutic gene may encode antisense *ras*, antisense *myc*, antisense *raf*, antisense *erb*, antisense *src*, antisense *fms*, antisense *jun*, antisense *trk*, antisense *ret*, antisense *gsp*, antisense *hst*, antisense *bcl* antisense *abl*, Rb, CFTR, p16, p21, p27, p57, p73, C-CAM, APC, CTS-1, zac1, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF, mda-7, thymidine kinase or p53. In an even more preferred embodiment, the therapeutic gene is p53. One of the most frequent abnormalities resulting in human cancer are mutations in p53, thus the ability to replace a deficient p53 gene using the present invention is highly desirable.

In another particular embodiment of the present invention, the adenovirus is harvested by steps that include lysing the producer cells by means other than freeze-thaw. The reason for this is that the freeze-thaw method is somewhat cumbersome and not particularly suited to production of commercial quantities. In preferred embodiments the producer cells are lysed by means of detergent lysis or autolysis. The harvesting of the adenovirus by detergent lysis and autolysis results in a much higher virus recovery than the freeze-thaw process and is therefore an improvement in the large scale production of adenoviruses.

In a particular embodiment of the present invention the purified recombinant adenovirus has one or more of the following properties. For example, the property may be a virus titer of between about 1×10^9 and about 1×10^{13} pfu/ml, a virus particle concentration between about 1×10^{10} and about 2×10^{13} particles/ml, a particle:pfu ratio between about 10 and about 60, less than 50 ng BSA per 1×10^{12} viral particles, between about 50 pg and 1 ng of contaminating human DNA per 1×10^{12} viral particles or a single HPLC elution peak consisting essentially of 98 to 99.9% of the area under the peak. These criteria select for a highly purified adenovirus.

To further impose limits on the purification process of the adenovirus, between about 5×10^{14} and 1×10^{18} viral particles are desired. In addition, one or more of the following properties further improve the selection for high purity adenovirus particles. For example the property may be a virus titer of between about 1×10^9 and about 1×10^{13} pfu/ml, more preferably 1×10^{11} and about 1×10^{13} pfu/ml, and most preferably 1×10^{12} and about 1×10^{13} pfu/ml. Further, a virus particle concentration between about 1×10^{10} and about 2×10^{13} particles/ml, more preferably 1×10^{11} and about 2×10^{13} particles/ml, and most preferably 1×10^{12} and about 1×10^{13} particles/ml.

Additionally, a particle:pfu ratio between about 10 and about 60, more preferably a particle:pfu ratio between about 10 and about 50, even more preferable a particle:pfu

ratio between about 10 and about 40, and most preferably a particle:pfu ratio between about 15 and about 40.

To limit the BSA concentration, it is preferable to have less than 50 ng BSA per 1 x 10^{12} viral particles, for example, between about 1 ng to 50 ng BSA per 1 x 10^{12} viral particles, and more preferably between about 5 ng and 40 ng of BSA per 1 x 10^{12} viral particles.

Low concentrations of DNA contamination are also desired. Thus, between about 50 pg and 1 ng of contaminating human DNA per 1 x 10^{12} viral particles is acceptable, even more preferable is between about 50 pg and 1000 pg of contaminating human DNA per 1 x 10^{12} viral particles, and most preferable is between about 100 pg and 1000 pg of contaminating human DNA per 1 x 10^{12} viral particles. Finally, an adenovirus that elutes as a single HPLC peak is desired, more preferably is an adenovirus that elutes as an HPLC peak that contains between about 98 and 99.99% of the total area under the peak.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and FIG. 1B. HPLC profiles of the viral solutions from production runs using medium perfusion rates characterized as "high" (FIG. 1A) and "low" (FIG. 1B).

FIG. 2. The HPLC profile of crude cell lysate (CCL) from CellCube™ (solid line A_{260}; dotted line A_{280}).

FIG. 3A, FIG. 3B, FIG 3C, FIG. 3D and FIG. 3E. The HPLC profiles of lysis solutions from CellCube™ using different detergents. FIG. 3A Thesit®. FIG. 3B Triton®X-100. FIG. 3C. NP-40®. FIG. 3D. Brij®80. FIG. 3E. Tween®20. Detergent concentration: 1% (w/v) lysis temperature: room temperature. (solid line A_{260}; dotted line A_{280}).

FIG. 4A and FIG. 4B. The HPLC profiles of virus solution before (FIG. 4A) and after (FIG. 4B) Benzonase treatment. (solid line A_{260}; dotted line A_{280}).

FIG. 5. The HPLC profile of virus solution after Benzonase treatment in the presence of 1M NaCl. (solid line A_{260}; dotted line A_{280}).

FIG. 6. Purification of AdCMVp53 virus under buffer A condition of 20mM Tris + 1mM $MgCl_2$ + 0.2M NaCl, pH=7.5.

FIG. 7. Purification of AdCMVp53 virus under buffer A condition of 20mM Tris + 1mM $MgCl_2$ + 0.2M NaCl, pH=9.0.

FIG. 8A, FIG. 8B, and FIG. 8C. HPLC analysis of fractions obtained during purification FIG. 8A fraction 3. FIG. 8B fraction 4, FIG. 8C fraction 8. (solid line A_{260}; dotted line A_{280}).

FIG. 9. Purification of AdCMVp53 virus under buffer A condition of 20mM Tris + 1mM $MgCl_2$ + 0.3M NaCl, pH=9.

FIG. 10A, FIG. 10B, FIG. 10C, FIG. 10D and FIG. 10E. HPLC analysis of crude virus fractions obtained during purification and CsCl gradient purified virus. FIG. 10A Crude virus solution. FIG. 10B Flow through. FIG. 10C. Peak number 1. FIG. 10D. Peak number 2. FIG. 10E. CsCl purified virus. (solid line A_{260}; dotted line A_{280}).

FIG. 11. HPLC purification profile from a 5cm id column.

FIG. 12. The major adenovirus structure proteins detected on SDS-PAGE.

FIG. 13. The BSA concentration in the purified virus as detected level of the western blot assay.

FIG. 14. The chromatogram for the crude cell lysate material generated from the CellCube™.

FIG. 15. The elution profile of treated virus solution purified using the method of the present invention using Toyopearl SuperQ resin.

FIG. 16A and **FIG. 16B.** HPLC analysis of virus fraction from purification protocol. FIG 16A HPLC profiles of virus fraction from first purification step. FIG.16B HPLC profiles of virus fraction from second purification. (solid line A_{260}; dotted line A_{280}).

FIG. 17. Purification of 1% Tween® harvest virus solution under low medium perfusion rate.

FIG. 18. HPLC analysis of the virus fraction produced under low medium perfusion rate.

FIG. 19A, FIG. 19B and FIG. 19C. Analysis of column purified virus. FIG. 19A SDS-PAGE analysis. FIG. 19B Western blot for BSA. FIG. 19C nucleic acid slot blot to determine the contaminating nucleic acid concentration.

FIG. 20A, FIG. 20B, FIG. 20C, FIG. 20D, FIG. 20E and FIG. 20F. Capacity study of the Toyopearl SuperQ 650M resin. FIG. 20A Flow through from loading ratio of 1:1. FIG. 20B. Purified virus from loading ratio of 1:1. FIG. 20C Flow through of loading ratio of 2:1. FIG. 20D. Purified virus from the loading ratio of 2:1. FIG. 20E Flow through from

loading ratio of 3:1. FIG. 20F. Purified virus from the loading ratio of 3:1. (solid line A_{260}; dotted line A_{280}).

FIG. 21. Isopycnic CsCl ultracentrifugation column purified virus.

FIG. 22A and FIG. 22B. The HPLC profiles of intact viruses present in the column purified virus. A. Intact virus B. Defective virus. (solid line A_{260}; dotted line A_{280}).

FIG. 23. A production and purification flow chart for AdCMVp53

FIG. 24. Kinetics of virus release in the supernatant in a 4x100 CellCube™.

FIG. 25. Chromatogram using Source 15Q resin for purification.

FIG. 26. HPLC profile of purified Ad5CMV-p53 product from Source 15Q resin.

FIG. 27A and FIG. 27B. Comparison of bioactivity of original process vs. optimized process to produce Ad5CMV-p53 product.

FIG. 28. Production and Purification flow chart for Ad5CMV-p53 optimized process.

FIG. 29. Lyophilization cycle for adenovirus formulations.

FIG. 30A and FIG. 30B. Storage stability data using secondary drying at 10^0 C without N_2 blanketing. FIG. 30A, secondary drying at 10^0 C without N_2 blanketing for formulation set 10; 6%-mannitol, 0.5% HAS, 1%-glycerol and different percentages of

sucrose in 10 mM-tris buffer (pH-7.5, 1 1mM $MgCl_2$). FIG. 30B, secondary drying at 10^0 C without N_2 blanketing for formulation set 11; 5%-mannitol, 0.5% HAS, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH-7.5, 1mM $MgCl_2$).

FIG. 31A and FIG. 31B. Storage stability data using secondary drying at 30^0 C without N_2 blanketing. Fig 31A, secondary drying at 30^0 C without N_2 blanketing for formulation set 10; 6%-mannitol, 0.5% HAS, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH-7.5, 1 1mM $MgCl_2$). FIG. 31B, secondary drying at 30^0 C without N_2 blanketing for formulation set 11; 5%-mannitol, 0.5% HAS, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH-7.5, 1mM $MgCl_2$).

FIG. 32A and FIG. 32B. Storage stability data using secondary drying at 30^0 C with N_2 blanketing. FIG. 32A, secondary drying at 30^0 C with N_2 blanketing for formulation set 10; 6%-mannitol, 0.5% HAS, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH-7.5, 1 1mM $MgCl_2$).. FIG. 32B, secondary drying at 30^0 C with N_2 blanketing for formulation set 11; 5%-mannitol, 0.5% HAS, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH-7.5, 1mM $MgCl_2$).

FIG. 33. Stability data for liquid formulation set #1. G: glycerol; S: sucrose; PEG: PEG-3500; T2: trehalose. Glycerol is in PBS buffer (10%). Other formulations are in 10 mM-tris buffer with 0.15 M-NaCl and mM-$MgCl_2$ (pH-8.2).

FIG. 34A and FIG. 34B. Stability data for liquid formulation set #2. Excipients are in 10 mM-tris buffer (pH-8.2) which consists of 0.5% glycerol, 0.15 M-NaCl and 1mM $MgCl_2$. The formulations are stored at 4°C and room temperature under nitrogen.

FIG. 35. Stability data for liquid formulation set #3.

FIG. 36. Stability data for liquid formulation set #4. Excipients are in 10 mM-tris buffer (pH-8.2) which consists of 1% glycerol, 0.15 M-NaCl and 1 mM $MgCl_2$. The formulations with virus are stored at 4°C and room temperature under nitrogen.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

It has been shown that adenoviral vectors can successfully be used in eukaryotic gene expression and vaccine development. Recently, animal studies have demonstrated that recombinant adenovirus could be used for gene therapy. Successful studies in administering recombinant adenovirus to different tissues have proven the effectiveness of adenoviral vectors in therapy. This success has led to the use of such vectors in human clinical trials. There now is an increased demand for the production of adenoviral vectors to be used in various therapies. The techniques currently available are insufficient to meet such a demand. The present invention provides methods for the production of large amounts of adenovirus for use in such therapies.

The present invention involves a process that has been developed for the production and purification of a replication deficient recombinant adenovirus. The production process is based on the use of a cell culture bioreactor for cell growth and virus production. After viral infection of the producer cells, virus can be harvested by any number of methods, including virus autolysis or chemical lysis. The harvested crude virus solution can then be purified using a single ion exchange chromatography run, after concentration/diafiltration and nuclease treatment to reduce the contaminating nucleic acid concentration in the crude virus solution. The column purified virus has equivalent purity relative to that of virus purified by cesium banding. The total process recovery of the virus product is 70% ± 10%. This is a significant improvement over the results reported by Huyghe *et al.* (1996). Compared to

double CsCl gradient ultracentrifugation, column purification has the advantage of being more consistent, scaleable, validatable, faster and less expensive. This new process represents a significant improvement in the technology for manufacturing of adenoviral vectors for gene therapy.

Therefore, the present invention is designed to take advantage of these improvements in large scale culturing systems and purification for the purpose of producing and purifying adenoviral vectors. The various components for such a system, and methods of producing adenovirus therewith, are set forth in detail below.

1. Host Cells

A) Cells

In a preferred embodiment, the generation and propagation of the adenoviral vectors depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Adenovirus serotype 5 (Ad5) DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the Ad genome (Jones and Shenk, 1978), the current Ad vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991; Bett *et al.*, 1994).

A first aspect of the present invention is the recombinant cell lines which express part of the adenoviral genome. These cells lines are capable of supporting replication of adenovirus recombinant vectors and helper viruses having defects in certain adenoviral genes, *i.e.*, are "permissive" for growth of these viruses and vectors. The recombinant cell also is referred to as a helper cell because of the ability to complement defects in, and support replication of, replication-incompetent adenoviral vectors. The prototype for an adenoviral helper cell is the 293 cell line, which contains the adenoviral E1 region. 293 cells support the replication of adenoviral vectors lacking E1 functions by providing *in trans* the E1-active

elements necessary for replication. Other cell lines which also support the growth of adenoviruses lacking E1 function include PER.C6 (IntroGene, NL), 911 (IntroGene, NL), and IT293SF.

Helper cells according to the present invention are derived from a mammalian cell and, preferably, from a primate cell such as human embryonic kidney cell. Although various primate cells are preferred and human or even human embryonic kidney cells are most preferred, any type of cell that is capable of supporting replication of the virus would be acceptable in the practice of the invention. Other cell types might include, but are not limited to Vero cells, HeLa cells or any eukaryotic cells for which tissue culture techniques are established as long as the cells are adenovirus permissive. The term "adenovirus permissive" means that the adenovirus or adenoviral vector is able to complete the entire intracellular virus life cycle within the cellular environment.

The helper cell may be derived from an existing cell line, *e.g.*, from a 293 cell line, or developed *de novo*. Such helper cells express the adenoviral genes necessary to complement *in trans* deletions in an adenoviral genome or which support replication of an otherwise defective adenoviral vector, such as the E1, E2, E4, E5 and late functions. A particular portion of the adenovirus genome, the E1 region, has already been used to generate complementing cell lines. Whether integrated or episomal, portions of the adenovirus genome lacking a viral origin of replication, when introduced into a cell line, will not replicate even when the cell is superinfected with wild-type adenovirus. In addition, because the transcription of the major late unit is after viral DNA replication, the late functions of adenovirus cannot be expressed sufficiently from a cell line. Thus, the E2 regions, which overlap with late functions (L1-5), will be provided by helper viruses and not by the cell line. Typically, a cell line according to the present invention will express E1 and/or E4.

As used herein, the term "recombinant" cell is intended to refer to a cell into which a gene, such as a gene from the adenoviral genome or from another cell, has been introduced. Therefore, recombinant cells are distinguishable from naturally-occurring cells which do not contain a recombinantly-introduced gene. Recombinant cells are thus cells having a gene or genes introduced through "the hand of man."

Replication is determined by contacting a layer of uninfected cells, or cells infected with one or more helper viruses, with virus particles, followed by incubation of the cells. The formation of viral plaques, or cell free areas in the cell layer, is the result of cell lysis caused by the expression of certain viral products. Cell lysis is indicative of viral replication.

Examples of other useful mammalian cell lines that may be used with a replication competent virus or converted into complementing host cells for use with replication deficient virus are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, HepG2, 3T3, RIN, MDCK and A549 cells.

B) Growth in selection media

In certain embodiments, it may be useful to employ selection systems that preclude growth of undesirable cells. This may be accomplished by virtue of permanently transforming a cell line with a selectable marker or by transducing or infecting a cell line with a viral vector that encodes a selectable marker. In either situation, culture of the transformed/transduced cell with an appropriate drug or selective compound will result in the enhancement, in the cell population, of those cells carrying the marker.

Examples of markers include, but are not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk-*, *hgprt-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used

as the basis of selection for *dhfr*, that confers resistance to methotrexate; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

C) Growth in serum weaning

Serum weaning adaptation of anchorage-dependent cells into serum-free suspension cultures have been used for the production of recombinant proteins (Berg, 1993) and viral vaccines (Perrin, 1995). There have been few reports on the adaptation of 293A cells into serum-free suspension cultures until recently. Gilbert reported the adaptation of 293A cells into serum-free suspension cultures for adenovirus and recombinant protein production (Gilbert, 1996). A similar adaptation method had been used for the adaptation of A549 cells into serum-free suspension culture for adenovirus production (Morris *et al.*, 1996). Cell-specific virus yields in the adapted suspension cells, however, are about 5-10-fold lower than those achieved in the parental attached cells.

Using the similar serum weaning procedure, the inventors have successfully adapted the 293A cells into serum-free suspension culture (293SF cells). In this procedure, the 293 cells were adapted to a commercially available 293 media by sequentially lowering down the FBS concentration in T-flasks. Briefly, the initial serum concentration in the media was approximately 10% FBS DMEM media in T-75 flask and the cells were adapted to serum-free IS 293 media in T-flasks by lowering down the FBS concentration in the media sequentially. After 6 passages in T-75 flasks the FBS% was estimated to be about 0.019% in the 293 cells. The cells were subcultured two more times in the T flasks before they were transferred to spinner flasks. The results described herein below show that cells grow satisfactorily in the serum-free medium (IS293 medium, Irvine Scientific, Santa Ana, CA). Average doubling time of the cells were 18-24 h achieving stationary cell concentrations in the order of 4-10 $\times$ 10^6 cells/ml without medium exchange.

D) Adaptation of cells for Suspension Culture

Two methodologies have been used to adapt 293 cells into suspension cultures. Graham adapted 293A cells into suspension culture (293N3S cells) by 3 serial passages in nude mice (Graham, 1987). The suspension 293N3S cells were found to be capable of supporting E1 adenoviral vectors. However, Garnier *et al.* (1994) observed that the 293N3S cells had a relatively long initial lag phase in suspension, a low growth rate, and a strong tendency to clump.

The second method that has been used is a gradual adaptation of 293A cells into suspension growth (Cold Spring Harbor Laboratories, 293S cells). Garnier *et al.* (1994) reported the use of 293S cells for production of recombinant proteins from adenoviral vectors. The authors found that 293S cells were much less clumpy in calcium-free media and a fresh medium exchange at the time of virus infection could significantly increase the protein production. It was found that glucose was the limiting factor in culture without medium exchange.

In the present invention, the 293 cells adapted for growth in serum-free conditions were adapted into a suspension culture. The cells were transferred in a serum-free 250 mL spinner suspension culture (100 mL working volume) for the suspension culture at an initial cell density of between about 1.18E+5 vc/mL and about 5.22E+5 viable cells/mL. The media may be supplemented with heparin to prevent aggregation of cells. This cell culture systems allows for some increase of cell density whilst cell viability is maintained. Once these cells are growing in culture, the cells are subcultured in the spinner flasks approximately 7 more passages. It may be noted that the doubling time of the cells is progressively reduced until at the end of the successive passages the doubling time is about 1.3 day, *i.e.* comparable to 1.2 day of the cells in 10% FBS media in the attached cell culture. In the serum-free IS 293

media supplemented with heparin almost all the cells existed as individual cells not forming aggregates of cells in the suspension culture.

2. Cell Culture Systems

In any cell culture system, there is a characteristic growth pattern following inoculation that includes a lag phase, an accelerated growth phase, an exponential or "log" phase, a negative growth acceleration phase and a plateau or stationary phase. The log and plateau phases give vital information about the cell line, the population doubling time during log growth, the growth rate, and the maximum cell density achieved in plateau. In the log phase, as growth continues, the cells reach their maximum rate of cell division. Numbers of cells increase in log relationship to time. During this period of most active multiplication, the logarithms of the numbers of cells counted at short intervals, plotted against time, produce a straight line. By making one count at a specified time and a second count after an interval during the log phase of growth and knowing the number of elapsed time units, one can calculate the total number of cell divisions or doublings, and both the growth rate and generation time. Within a few hours or days after the commencement of the log phase, the rate of cell division begins to decline and some of the cells begin to die. This is reflected on the growth curve by a gradual flattening out of the line. Eventually the rate of cells dying is essentially equal to the rate of cells dividing, and the total viable population remains the same for a period of time. This is known as the stationary or plateau phase and is represented on the growth curve as a flattening out of the line where the slope approaches zero.

Measurement of the population doubling time can be used to quantify the response of the cells to different inhibitory or stimulatory culture conditions such as variations in nutrient concentration, hormonal effects, or toxic drugs. It is also a good monitor of the culture during serial passage and enables the calculation of cell yields and the dilution factor required at subculture.

The population doubling time is an average figure and describes the net result of a wide range of cell division rates, including zero, within the culture. The doubling time will also differ with varying cell types, culture conditions, and culture vessels. Single time points are unsatisfactory for monitoring growth when the shape of the cell growth curve is not known. Thus it is important to determine the growth curve for each cell type being used in the conditions that are being used for the cell culture. Typical growth curves are sigmoidal in shape, with the first part of the curve representing the lag phase, the center part of the curve representing the log phase, and the last part of the curve representing the plateau phase. The log phase is when the cells are growing at the highest rate, and as the cells reach their saturation density, their growth will slow and the culture will enter the plateau phase. A detailed description of cell culture techniques and theory can be found in Freshney, 1992 and Freshney, 1987.

An important aspect of the present invention is infection of the producer cells with recombinant adenovirus at an appropriate time to achieve maximal virus production. The inventors have found that maximal virus production is obtained when the producer cells are infected between about when the cells reach the first inflection point on the log phase of the cell growth curve, i.e. mid-log phase, and before the 2^{nd} inflection point on the plateau phase of the cell growth curve, i.e. mid-plateau phase. This range can be determined easily for any cell type and any culture conditions with any cell culturing apparatus. The inflection points on a cell growth curve are when the shape of the line changes from a convex to a concave shape, or from a concave to a convex shape.

For most growth curves plotted on semi-log scales, the log phase of growth can be approximately represented by a linear increase in the slope of the line over time. That is, at any short interval between two points on the line of the logarithmic phase of the curve, the

log of cell number is increasing in a linear fashion relative to time. Thus mid log phase can be approximately defined as the point or interval within the log phase in which the cells are dividing at their maximal rate, and the increase in logs of cell number is linear with respect to time. Late log phase can be defined as approximately the point or interval of time in which the rate of cell division has slowed, and the log of number of cells is no longer increasing in a linear fashion with respect to time. When looking at a growth curve, this area would be represented by gradual falling or flattening of the slope of the line. At early stationary phase, the rate of cell growth is decreasing and getting nearer the rate of cell death, and thus the slope of the line on the growth curve is even less than that at late log phase. At mid-stationary phase, the rate of cell growth is approximately equal to the rate of cell division and thus the line on the growth curve is relatively flat and has a slope approaching zero. It will be understood that the skilled artisan can formulate growth curves for any such cell line and identify the aforementioned regions on the curve.

The ability to produce infectious viral vectors is increasingly important to the pharmaceutical industry, especially in the context of gene therapy. Over the last decade, advances in biotechnology have led to the production of a number of important viral vectors that have potential uses as therapies, vaccines and protein production machines. The use of viral vectors in mammalian cultures has advantages over proteins produced in bacterial or other lower lifeform hosts in their ability to post-translationally process complex protein structures such as disulfide-dependent folding and glycosylation.

Development of cell culture for production of virus vectors has been greatly aided by the development in molecular biology of techniques for design and construction of vector systems highly efficient in mammalian cell cultures, a battery of useful selection markers, gene amplification schemes and a more comprehensive understanding of the biochemical and

cellular mechanisms involved in procuring the final biologically-active molecule from the introduced vector.

Frequently, factors which affect the downstream (in this case, beyond the cell lysis) side of manufacturing scale-up were not considered before selecting the cell line as the host for the expression system. Also, development of bioreactor systems capable of sustaining very high density cultures for prolonged periods of time have not lived up to the increasing demand for increased production at lower costs.

The present invention will take advantage of the recently available bioreactor technology. Growing cells according to the present invention in a bioreactor allows for large scale production of fully biologically-active cells capable of being infected by the adenoviral vectors of the present invention. By operating the system at a low perfusion rate and applying a different scheme for purification of the infecting particles, the invention provides a purification strategy that is easily scaleable to produce large quantities of highly purified product.

Bioreactors have been widely used for the production of biological products from both suspension and anchorage dependent animal cell cultures. The most widely used producer cells for adenoviral vector production are anchorage dependent human embryonic kidney cells (293 cells). Bioreactors to be developed for adenoviral vector production should have the characteristic of high volume-specific culture surface area in order to achieve high producer cell density and high virus yield. Microcarrier cell culture in stirred tank bioreactor provides very high volume-specific culture surface area and has been used for the production of viral vaccines (Griffiths, 1986). Furthermore, stirred tank bioreactors have industrially been proven to be scaleable. The multiplate Cellcube™ cell culture system manufactured by Corning-Costar also offers a very high volume-specific culture surface area. Cells grow on

both sides of the culture plates hermetically sealed together in the shape of a compact cube. Unlike stirred tank bioreactors, the Cellcube™ culture unit is disposable. This is very desirable at the early stage production of clinical product because of the reduced capital expenditure, quality control and quality assurance costs associated with disposable systems. In consideration of the advantages offered by the different systems, both the stirred tank bioreactor and the Cellcube™ system were evaluated for the production of adenovirus.

Table 1 list several exemplary techniques for cell culturing and viral particle production. Currently, there are no methods employed that result in both high purity and a high number of viral particles. Thus, the following methods are considered in combination with the large scale process for the production and purification of adenovirus described in the present invention.